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Lipid Screening by Thin Layer Chromatography

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A thin layer chromatographic method for the analysis of lipids in serum is described. Sterol esters, triglycerides, free sterols, free fatty acids, and phospholipids can be separated. After charring by heating with 10% sulphuric acid and clearing with a clearing solution, the thin layer chromatograms can be scanned with the Analytrol Scanner. This method permits phenotyping of hyperlipoproteinaemia, and a rapid evaluation of the ratio of the five most important lipid components can be made.

Eine dünnschichtchromatographische Methode zur Bestimmung der Serumlipide wird beschrieben. Sterinester, Triglyceride, freies Cholesterin, freie Fettsäuren und Phospholipide können bestimmt werden. Nach Verkohlen durch Erhitzen mit 10proz. Schwefelsäure und Aufhellen mit einer Transparenzbad-Lösung können die Dünnschichtchromatogramme mit dem Analytrol Scanner ausgewertet werden. Die Methode erlaubt, den Phänotyp von Hyperlipoproteinämien festzustellen. Das Verhältnis der fünf bedeutendsten Lipidkomponenten kann in kurzer Zeit bestimmt werden.

In a previous article (1) we described methods for the routine classification of the most common types of primary hyperlipoproteinaemia according to Frederickson and Coworkers (2). These methods included the determination of cholesterol, triglycerides, and total lipids, as well as an electropherographic procedure for the analysis of the lipoproteins on cellulose acetate. Recently, van Gent (3) described a method for the separation and microdetermination of lipids by thin layer chromatography followed by densitometry, based on the work of Peifer (4). With this method it is possible to determine the relative amounts of sterol esters, triglycerides, free sterols, free fatty acids, and total phospholipids in serum.

This article reported the densitometric analysis of lipid extracts from only 6 sera. To explore wider applications, we applied the method to a larger number of sera, evaluated the method statistically, compared the relative amounts of sterol ester, triglycerides, free sterols and total phospholipids with those found by routine methods, and attempted to determine whether it was possible to arrive at a classification of types II, III, and IV according to Frederickson by means of thin layer chromatography.

Methods

Quantitative analysis of the blood lipids

Total lipids were determined according to Drevon and Schmit (5, 1).

Free and total cholesterol were determined according to Sperry and Webb (6). Sterol esters were calculated from the difference between free and total cholesterol, multiplying by a factor 1.7. For the determination of the triglycerides we used the enzymatic procedure of Eggstein and Kreutz (7) (Boehringer Mannheim GmbH, W. Germany). Phospholipids were determined according to Wachter (8) (mg/100 m*l* lipid-phosphorus × 25 = mg/100 m*l* phospholipids). The free fatty acids were not determined quantitatively, since they have no relevance for classification according to Frederickson.

Preparation of the serum for thin layer chromatography

For the chromatographic separation, the lipid mixture dissolved in chloroform is applied to a thin-layer slide. The serum lipid mixtures were prepared by a zinc precipitation and an extraction with Folch solvents according to Friedman (9).

Reagents

Acid zinc reagent, prepared by 35:1000 dilution of a stock solution containing 100 g $ZnSO_4 \cdot 7\ H_2O$ and 250 mVal H_2SO_4/l.

*NaOH, 0.375*N, adjusted so that 20.0 m*l* of acid zinc reagent requires 5.0 ± 0.05 m*l* to neutralize to phenolphthalein.

Chloroform p. a.

Methanol p. a.

Method

To 10 m*l* zinc reagent in a centrifuge tube fitted with a glass stopper, add 0.5 m*l* serum. After mixing on a Vortex mixer, add 0.5 m*l* of 0.375N NaOH.

Stopper the tube, mix thoroughly, and let stand at room temperature for 5 min. Centrifuge at 2500—3000 rpm for 10 min. Decant and discard the supernatant. Drain the tube over filter paper for 5 min. Mix the precipitate thoroughly with 12.5 m*l* distilled water and centrifuge again. After discarding the supernatant and draining as above, add 5 m*l* methanol followed by 10 m*l* chloroform and mix the precipitate vigorously for 1 minute. After centrifugation, decant the clear supernate into another centrifuge tube.

Evaporate the extract to dryness in a 50° waterbath under a stream of nitrogen. Cool as soon as the last traces of solvent have disappeared, and dissolve the residue in chloroform, using 0.05 m*l* chloroform per 100 mg/100 m*l* total lipid. The total lipids of the extracted serum have, of course, been determined before the extraction, by the method of Drevon and Schmit (5). Close the centrifuge tube with a glass stopper.

The serum extract is now ready for thin layer chromatography.

Chromatographic Procedure

For the chromatographic separation of the lipids in the serum extract, a modification of the method of van Gent (3) is used. Using a special clearing solution (see under Reagents), it is possible to scan the chromatograms with the Analytrol scanner.

Materials

1. Glass slides, 76 × 26 mm, to which the silica gel is applied.
2. 2 hot-plates, one kept at 100° and the other at 200°.
3. A small chromatography chamber (3.5 × 5.5 × 10 cm) for the development of the chromatograms. This chamber must be fitted with a close-fitting lid. The best results are obtained with these small chambers and the small thin layer plates if the lid ist completely sealed with tape.
4. A pair of tweezers.
5. A fine capillary, drawn out to a small point, for applying the extract to the silica gel. About 10 μl extract is to be applied.
6. A spray gun (Shandon).
7. Analytrol Scanner (Beckman Model R. B.).

Reagents

1. Silica gel (Silikagel D. O., Camag).
2. Chloroform p. a.
3. Hexane p. a.
4. Sulphuric acid 10%.
5. Clearing solution for making the slides transparent: a mixture of 2 parts Nitro Rubbol Metal Varnish, Colorless (Sikkens N. V. Holland) and 1 part ethyl acetate.

Method

Make a suspension of 15 g silica gel in 90 m*l* chloroform. With stirring, dip two slides into the suspension, holding them together with the tweezers.

Move the slides gently to and fro and then lift them out of the suspension. Separate the slides and dry them at room temperature for several minutes, after which they are ready for use. Apply about 10 μl of the serum extract to the thin layer slides by means of a fine capillary. The extract is applied as a series of single spots, so close together that they form a band.

Place the slides in the chromatography chamber, which contains chloroform. As soon as the front has reached the middle of the slides, remove and dry them for about 30 seconds at room temperature in the air. Then place them in a second chromatography chamber containing hexane-chloroform 3:1.

When the front approaches the top of the slides, take them out of the chamber and dry them as before. Next, spray the slides with 10% sulphuric acid, using the spray gun. Heat the slides for 30 to 60 seconds on the hot-plate set at 100° and then on the second hot-plate at 200° for 15 minutes. Black bands indicating the charred lipids now appear. Cool the slides. To make the slides transparent, flow 2.5 m*l* of the clearing solution onto each slide, dry for 5 to 10 min. at room temperature in the air, and apply 1 m*l* clearing solution. Dry again for 5 to 10 min. and apply 1 m*l* clearing solution.

Let the slides dry overnight in the air, after which they will be transparent and can be scanned with the Analytrol Scanner. Moreover, the layer can be taken off the slide, making storage more convenient.

All experiments with the thin layer procedure were carried out in duplicate.

Results

The chromatographic analysis

Figure 1 gives an example of a thin layer chromatogram.

As shown by VAN GENT (3), the number of densitometric units per microgram of lipid is dependent on the composition of the lipid.

To calculate the composition of a mixture, the densitometric values obtained have to be multiplied by a correction factor specific for each component. In our experiments this correctionfactor was determined by comparing the percentages found by chromatographic analysis with the percentages found by the routine method. The correction factors, thus obtained, are given in Table 1.

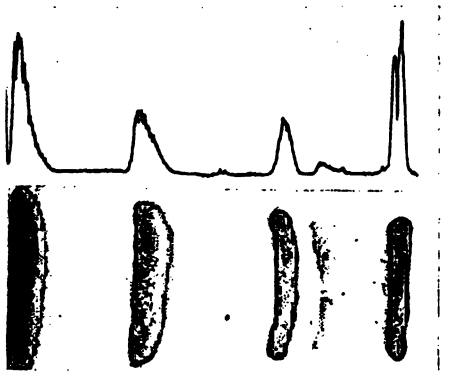

Fig. 1
Thin layer chromatogram of a serum extract. From left to right: bands and peaks of sterol esters, triglycerides, free sterols free fatty acids, and phospholipids

Tab. 1
Correction factors for the calculation of the lipid composition from the densitometric determination. The densitometric values must be multiplied by these factors

Sterol esters	1.40
Triglycerides	1.91
Free sterols	1.00
Phospholipids	1.96
Free Fatty Acids	1.95

Tab. 2
Standard deviation of the chromatographic procedure (40 samples of the same extract from a pooled serum)

	Relative Peak Area	
Sterol esters	37%	S. D. 2.5%
Triglycerides	26%	S. D. 2.2%
Free sterols	12%	S. D. 1.6%
Phospholipids	24%	S. D. 3.1%
Free Fatty Acids	1%	S. D. 0.7%

Tab. 3
Standard deviation of the extraction procedure (40 samples of different extracts from a pooled serum)

	Relative Peak Area	
Sterol esters	40%	S. D. 3.1%
Triglycerides	21%	S. D. 1.7%
Free sterols	8%	S. D. 0.2%
Phospholipids	27%	S. D. 2.3%
Free Fatty Acids	4%	S. D. 0.3%

To evaluate the standard deviation of the chromatographic method, 40 chromatograms of the same extract of a pooled serum were made. The results are shown in Table 2.

In a second experiment we checked the reproducibility of the extraction procedure. Forty extracts were made from a pooled serum and chromatographed. The results are shown in Table 3.

As can be seen from Tables 2 and 3, the reproducibility of the chromatographic and extraction procedure is good.

Comparison between chromatographic and routine methods

To evaluate the chromatographic procedure further, we compared the relative amounts of sterol esters, triglycerides, free sterols, and phospholipids found by chromatography and the relative amounts found by routine methods (see under Quantitative analysis of the blood lipids).

The results are shown in Table 4. The correlation is significant in all cases ($p < 0.001$).

Tab. 4
Comparison between the relative amounts of sterol esters, triglycerides, free sterol, and phospholipids obtained by chromatography (x) and quantitative determinations (y) (90 sera)

	y = bx + a	r	p
Sterol esters	y = 0.632x + 14.6	0.664	< 0.001
Triglycerides	y = 0.923x + 1.6	0.875	< 0.001
Free sterols	y = 0.468x + 4.2	0.798	< 0.001
Phospholipids	y = 0.403x + 16.6	0.476	< 0.001

r = correlation coefficient p = level of significance of r

The correlation is poorest for the phospholipids; this was to be expected, because the phospholipid band remains at the place where the sample is applied to the chromatogram, and the thin layer is slightly disturbed by the application. This reduces the accuracy of the densitometric evaluation.

Classification of the most common types

After checking the reproducibility of the chromatographic procedure and comparing this procedure with classical methods, we attempted to arrive at a classification of the most common types according to Frederickson on the basis of thin layer chromatography. As shown in our previous article (1), this can be done satisfactorily with the cholesterol/triglyceride ratio and the β lipoprotein/pre β lipoprotein ratio (electrophoretic procedure). Table 5 shows the averages and ranges of the lipid percentages of a number of sera, obtained by normal quantitative methods. Table 6 shows the results with thin layer chromatography.

As can be seen, there is a good agreement between the values in Tables 5 and 6. The cholesterol/triglyceride ratio, total cholesterol estimated as free sterols + sterol esters $\times$ 0.59, is especially suitable for phenotyping hyperlipoproteinaemia. Table 7 gives the values for the three different ways of phenotyping hyperlipoproteinaemia: the β lipoprotein/pre β liproprotein ratio, the cholesterol/triglyceride ratio, and the cholesterol/triglyceride ratio obtained by thin layer chromatography. There is a very good agreement indeed between the results of routine methods and thin layer chromatography.

Tab. 7
Phenotyping hyperlipoproteinaemia
Comparison of thin layer chromatography with routine methods

	Type II	Type III	Type IV
Cholesterol/triglyceride ratio (Postma and Stroes (1))	> 1.5	0.7—1.5	0.3—0.7
Cholesterol/triglyceride ratio (see Table 5)	> 1.6	0.8—1.7	0.3—0.7
Cholesterol/triglyceride ratio (see Table 6)	> 1.5	0.7—1.6	0.4—0.6
β lipoprotein/pre β lipoprotein ratio (Postma and Stroes (1))	> 2	0.7—2.0	< 0.7

Tab. 5
Lipid analysis by routine quantitative methods
Percentage of total lipids
(Average and range)

Type Number of patients	II 25	III 21	IV 6	Normals 23
Sterol esters	45 (36—52)	38 (30—48)	29 (22—35)	39 (30—48)
Triglycerides	15 (9—20)	26 (20—38)	44 (38—62)	19 (11—30)
Free sterols	9 (6—19)	8 (6—10)	6 (6—8)	7 (4—9)
Phospholipids	29 (22—36)	25 (22—28)	21 (14—28)	31 (22—36)
Total sterols	35 (30—40)	30 (24—37)	24 (18—28)	30 (22—36)
Cholesterol/triglyceride ratio	2.6 (1.6—4.3)	1.2 (0.8—1.7)	0.6 (0.3—0.7)	

Tab. 6
Lipid analysis by thin layer chromatography
Relative peak area (corrected by multiplication with charring factor)
(Average and range)

Type Number of patients	II 25	III 21	IV 6	Normals 23
Sterol esters	45 (33—54)	37 (32—44)	29 (24—32)	41 (33—49)
Triglycerides	15 (6—21)	27 (22—36)	41 (37—46)	20 (11—33)
Free sterols	9 (5—33)	7 (5—10)	5 (5—6)	7 (3—9)
Phospholipids	28 (20—37)	26 (22—29)	22 (18—25)	30 (22—38)
Total sterols	35 (30—52)	29 (25—36)	22 (19—24)	31 (24—36)
Cholesterol/triglyceride ratio	2.7 (1.5—8.5)	1.1 (0.7—1.6)	0.5 (0.4—0.6)	

Tab. 8

Some values from sera analyses for hyperlipoproteinaemia by routine quantitative methods (QM) and by thin layer chromatography (TLC)

	Total Lipids mg/100 ml	Sterol esters			Triglycerides			Free sterols			Phospholipids			Total Cholesterol*)		Cholesterol/ Triglycerides		β lipoprotein/ pre β lipoproteins (electrophoresis)
		mg/100 ml	% of Total Lipids	%	mg/100 ml	% of Total Lipids	%	mg/100 ml	% of Total Lipids	%	mg/100 ml	% of Total Lipids	%	% of Total Lipids	%			
	QM	QM	QM	TLC	QM	QM	TLC	QM	QM	TLC	QM	QM	TLC	QM	TLC	QM	TLC	
	A. Normal sera																	
1.	721	230	41	41	130	18	18	50	7	8	245	34	29	31	32	1.7	1.7	1.7
2.	738	325	44	45	85	12	15	62	8	8	258	35	30	34	34	3.0	2.2	2.2
3.	793	377	48	45	92	12	15	63	8	7	243	31	31	36	33	3.1	2.3	4.3
4.	692	316	46	49	92	13	11	48	6	6	233	34	31	34	35	2.5	3.1	6.0
	B. Type II																	
1.	1119	479	43	38	215	19	18	78	7	8	293	26	31	32	30	1.7	1.6	3.1
2.	886	398	45	46	114	13	11	83	9	8	315	36	34	36	35	2.8	3.2	3.7
3.	1055	452	43	44	177	17	12	95	9	8	335	32	35	34	34	2.0	2.9	5.9
4.	1100	495	45	49	201	18	12	96	9	8	333	30	29	35	37	1.9	3.0	8.5
	C. Type III																	
1.	1527	546	36	33	457	30	31	31	6	5	371	24	28	27	25	0.9	0.8	0.8
2.	1018	389	38	39	204	20	22	90	9	9	290	28	28	31	32	1.6	1.4	1.3
3.	1035	354	34	34	306	30	30	73	7	6	268	26	28	27	26	0.9	0.9	1.5
4.	1089	416	38	40	260	24	24	81	7	7	298	27	28	30	30	1.3	1.4	1.2
	D. Type IV																	
1.	2736	969	35	31	1140	42	44	206	8	5	378	14	18	28	23	0.7	0.5	<0.7
2.	1496	469	31	32	568	38	41	90	6	5	287	19	21	24	24	0.6	0.6	0.5
3.	1305	359	28	31	525	40	37	84	6	5	300	23	22	23	24	0.6	0.6	0.5
4.	2824	609	22	24	1744	62	46	158	6	6	470	17	20	18	19	0.3	0.4	0.1

*) Cholesterol from sterol esters and free sterols, estimated as free sterols + sterol esters × 0.59.
N. B. The percentages indicated for TLC are corrected percentages multiplied by the correction factor.

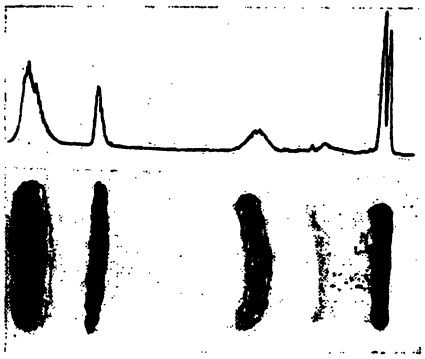

Fig. 2
Thin layer chromatogram of a type II hyperlipoproteinaemia

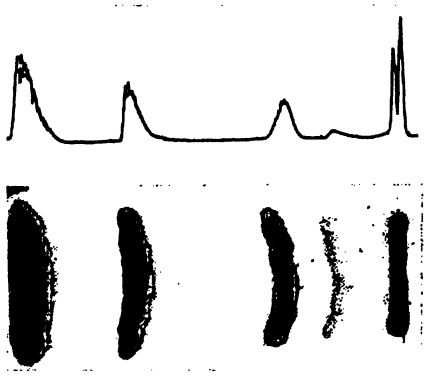

Fig. 3
Thin layer chromatogram of a type III hyperlipoproteinaemia

Lipid	Values found by quantitative determination		thin layer chromatography
	mg/100 ml	% of total lipids	% of total lipids
Total	1170	100	100
Sterol esters	470	40	42
Triglycerides	214	18	21
Free sterols	74	6	7
Phospholipids	318	27	28
Cholesterol/Triglyceride ratio		1.6	1.5
β lipoprotein/pre β lipoprotein ratio (Lipoprotein electrophoresis)		—	2.0

Lipid	Values found by quantitative determination		thin layer chromatography
	mg/100 ml	% of total lipids	% of total lipids
Total	1402	100	100
Sterol esters	530	38	40
Triglycerides	362	26	28
Free sterols	124	9	7
Phosphatids	323	23	22
Cholesterol/Triglyceride ratio		1.2	1.1
β lipoprotein/pre β lipoproteins ratio (Lipoprotein electroproteins)		—	2.0

Some examples of chromatograms and values

Since the results demonstrate that thin layer chromatography can be used for phenotyping hyperlipoproteinaemia, some examples will be useful. Figures 2, 3, and 4 give examples of types II, III, and IV of hyperlipoproteinaemia, respectively. Table 8 gives some values found in analyses of sera for hyperlipoproteinaemia, with comparison of the results of routine methods and thin layer chromatography. As can be seen from this Table, phenotyping can be done satisfactorily with thin layer chromatography using the criteria given in Table 8.

Conclusions

Thin layer chromatography offers a good method for phenotyping hyperlipoproteinaemia, in addition to the routine methods (determination of cholesterol and triglycerides and calculation of the cholesterol/triglyceride ratio) and lipid electrophoresis (β lipoprotein/pre β lipoprotein ratio).

When a rapid evaluation must be made of the ration of the five most important lipid components in serum, i. e. sterol esters, triglycerides, free sterols, free fatty acids and phospholipids, a thin layer chromatogram can give valuable information (Table 4).

We should like to thank Miss J. VAN CAPPELLEN for her very valuable technical assistance.

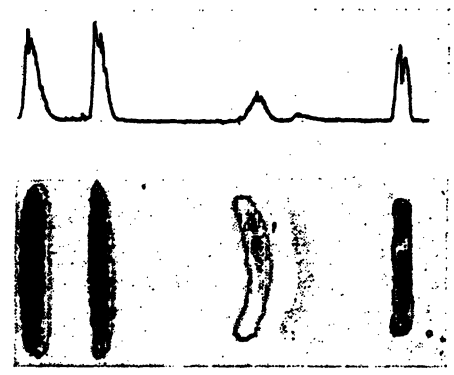

Fig. 4
Thin layer chromatogram of a type IV hyperlipoproteinaemia

Lipid	Values found by quantitative determination		thin layer chromatography
	mg/100 ml	% of total lipids	% of total lipids
Total	990	100	100
Sterol esters	292	30	30
Triglycerides	369	37	39
Free sterols	61	6	5
Phospholipids	270	27	23
Cholesterol/Triglyceride ratio		1.6	1.5
β lipoprotein/pre β lipoprotein ratio (Lipoprotein electrophoresis)		—	0.5

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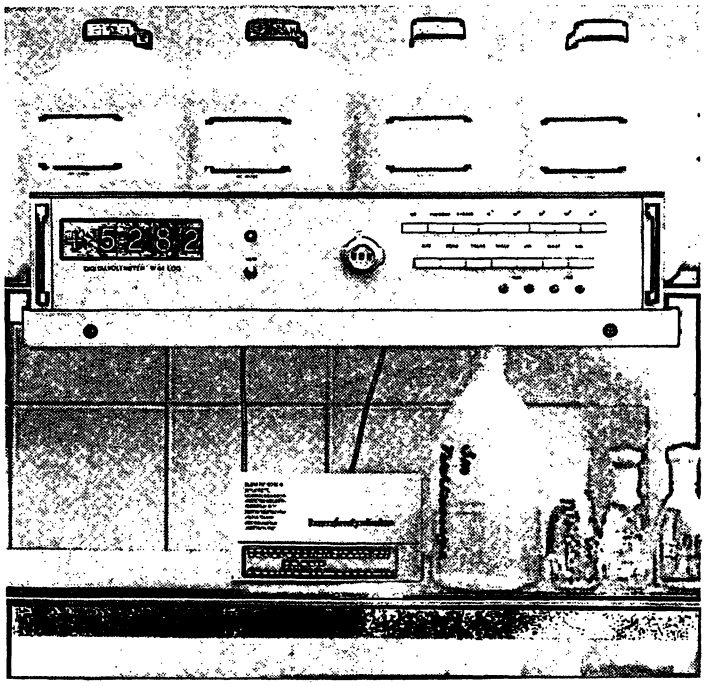

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